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Phosphorylation of Histone H1 by P-TEFb is a Necessary Step in Skeletal Muscle Differentiation

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Abstract

Positive transcription elongation factor b (P-TEFb), the complex of Cyclin T1 and CDK9, activates the transcription of many viral and eukaryotic genes at the point of mRNA elongation. The activity of P-TEFb has been implicated in the differentiation of a number of cell types, including skeletal muscle. In order to promote transcription, P-TEFb hyperphosphorylates RNA Pol II, thereby increasing its processivity. Our previous work identified histone H1 as a P-TEFb substrate during HIV-1 and immediate-early transcription. Here, we examine the role of P-TEFb phosphorylation of histone H1 during differentiation, using the myoblast cell line C2C12 as a model for skeletal muscle differentiation. We found that H1 phosphorylation is elevated in differentiating C2C12, and this phosphorylation is sensitive to P-TEFb inhibition. H1 phosphorylation was also necessary for the induction of three muscle marker genes that require P-TEFb for expression. Additionally, ChIP experiments demonstrate that H1 dissociates from muscle differentiation marker genes in C2C12 cells under active P-TEFb conditions. We determine that both P-TEFb activity and H1 phosphorylation are necessary for the full differentiation of C2C12 myoblasts into myotubes.

Transcription of many genes by RNA polymerase II (Pol II) is controlled at the step of mRNA elongation. The transcription elongation factor positive transcription elongation factor b (P-TEFb) participates in the activation of expression of many genes transcribed by Pol II. Comprised of the cyclin-dependent kinase CDK9 and Cyclin T1, P-TEFb phosphorylates the heptapeptide repeat of the largest subunit of RNA Pol II, increasing its processivity during elongation (Kim and Sharp, 2001; Peterlin and Price, 2006; Kohoutek, 2009). To enhance elongation, P-TEFb also phosphorylates other substrates such as the negative elongation factors DRB sensitivity-inducing factor and NELF (Ping and Rana, 2001). Our prior work has shown that P-TEFb also phosphorylates histone H1 to activate HIV-1 and immediate-early transcription (O'Brien et al., 2010). When phosphorylated by P-TEFb, H1 dissociates from DNA, allowing factors to access chromatin and Pol II to progress. We sought to know whether H1 phosphorylation is a requisite for transcription of other P-TEFb-regulated systems. As a model system for differentiation regulated by P-TEFb, we induced skeletal muscle differentiation in the myoblast cell line C2C12 and examined the activity of P-TEFb.

P-TEFb is indispensable for the transcription of HIV-1 genes as well as a number of cellular genes such as *c-fos, c-myc*, and the heat shock genes (Mancebo et al., 1997; Garber et al.,

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1998; Lis et al., 2000; Eissenberg et al., 2007). P-TEFb activity is also necessary for the differentiation of some tissues such as skeletal and cardiac muscle (Simone et al., 2002; Giacinti et al., 2006). In skeletal muscle, P-TEFb is recruited to differentiation-specific promoters by the major muscle transcription factors MyoD and MEF2 (Simone et al., 2002; De Falco and De Luca, 2006; Nojima et al., 2008). MyoD and the MEF2 family of transcription factors are responsible for the early expression of other muscle-specific transcription factors and expression of structural genes, respectively (Bergstrom et al., 2002; Blais et al., 2005). Because we found that P-TEFb phosphorylation of histone H1 is necessary for the transcription of HIV-1 genes and the cellular immediate early genes *c-fos* and *hsp70* (O'Brien et al., 2010).We hypothesized that P-TEFb could also modulate myogenic gene expression in C2C12 cells by phosphorylating H1. In particular, we chose to examine the early muscle marker gene and transcription factor myogenin, and the later-expressing markers muscle creatine kinase (MCK) and myosin heavy chain (MyHC), both essential to muscle contractile function. Using P-TEFb inhibition and H1 phosphorylation site mutants, qPCR, ChIP, and MyHC immunofluorescence, we show that P-TEFb phosphorylation of H1 is an important step in myogenic differentiation.

Experimental Procedures

Cell culture

C2C12 cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and were maintained at less than 50% confluency. To differentiate, cells were grown to >50% confluency, and media was changed to DMEM containing greater than 2% horse serum (Invitrogen).

Transfections and drug treatment

C2C12 cells were transfected with GFP-H1.1 constructs using Lipofectamine 2000 (Invitrogen). WT, T152A, S183A, T152A/S183A, and S183E GFP-H1.1 constructs were transfected at a concentration of 2.5 μg per well of six-well plates, and CDK9 and Myogenin Smartpool siRNAs (Dharmacon, Lafayette, CO) were transfected at a concentration of 50 nM. Both DNA and siRNA transfections were plated overnight, and differentiation was induced 24 h later. Flavopiridol (10–25 nM) or DMSO (0.1%) was added to cells growing in DMEM supplemented with FBS, incubated overnight, and differentiation was induced with DMEM supplemented with horse serum the following day. Flavopiridol was added to the media again during induction, which was carried out for 24 h before RNA or protein isolation.

qPCR

After induction of differentiation, RNA samples from C2C12 cells were isolated using Trizol (Invitrogen) and chloroform extraction followed by precipitation with isopropanol. cDNA samples were prepared using Superscript kit (Invitrogen) and EF1-α, myogenin, MCK, and MyHC transcripts were quantitated using AbsoluteBlue Sybr Green Mix (Thermo Fisher, Pittsburgh PA) and a Chromo 4 quantiative PCR (BioRad, Hercules, CA). Transcripts were quantified using a ΔΔC(t) method, normalizing to EF1-α. Primers for qPCR are listed in the Supporting Information Materials and Methods Section.

Cell lysates and Western blot

Total cell extracts for protein samples were taken after induction as described. Total cell extracts (2.5–5 μg) were run on 4–12% gradient gels, and blotted using anti-Cyclin T1, anti-CDK9, anti-histone H1, anti-myogenin, anti-MCK, anti-MyHC (all from Santa Cruz, Santa Cruz, CA), and anti-phospho-H1 (Millipore, Billerica, MA).

ChIP

ChIP was performed using anti-histone H1 (Millipore) and anti-histone H3 (Abcam, Cambridge, MA) antibodies essentially as described (Salma et al., 2004). Uninduced samples were plated on 15 cm dishes (Thermo Fisher, Pittsburgh, PA), while induced samples were plated on 10 cm dishes, and incubated in DMEM with 2% horse serum for 24 h. For each immunoprecipitation, 50 μg of protein was used in a 500 μl total volume with 1–3 μg of antibody. Absolute Blue Sybr Green PCR mix (Thermo) and 50 ng of each ChIP DNA sample were used for quantitation of binding at EF1-α, myogenin, MCK, and MyHC coding regions. Primer sets are listed in the Supporting Information Materials and Methods Section.

Immunofluorescence

C2C12 cells were treated or transfected as described above and incubated with differentiation media for 24–48 h, then fixed with 10% neutral buffered formalin (Sigma-Aldrich, Saint Louis, MO). Staining was then performed using anti-MyHC (Santa Cruz) and anti-rabbit secondary labeled with AlexaFluor 568 nm (Invitrogen). A scanning confocal microscope (Leica, Bannockburn, IL) was used to obtain fluorescence images.

Results

Inhibition of CDK9 activity in C2C12 cells prevents muscle marker gene expression

To evaluate the effect of P-TEFb inhibition on the differentiation of muscle cells, we treated C2C12 cells with flavopiridol, a specific inhibitor of CDK9, and depleted CDK9 using specific siRNAs. At 25 nM, flavopiridol can inhibit CDK9 but not the cell cycle related CDKs (Chao et al., 2000; Ali et al., 2009). We therefore plated C2C12 in growth media and treated cells with 25 nM flavopiridol or transfected C2C12 with CDK9 siRNA. To induce differentiation, the media was changed to DMEM containing 2% horse serum. We allowed differentiation to proceed for 24 h, and then prepared RNA extracts for qPCR. To evaluate a range of muscle-specific mRNAs, we performed qPCR for myogenin, a transcription factor that expresses shortly after induction of differentiation and activates expression of many muscle-specific genes, MCK, and MyHC, two proteins necessary for muscle function. When cells were pretreated with flavopiridol or CDK9 siRNA, the expression of these genes was not induced upon adding differentiation media (Fig. 1A,B). The effects of flavopiridol and CDK9 depletion were similar to the siRNA-mediated depletion of myogenin (Fig. 1A). To examine the specificity of this effect on transcription, we also measured cyclophilin A mRNA. Neither P-TEFb inhibition nor myogenin depletion affected cyclophilin A expression (Fig. 1C).

Flavopiridol treatment and CDK9 depletion were repeated and cell lysates were prepared to examine protein levels. Western blots also showed that H1 phosphorylation is increased in cells placed in differentiation media, and that both treatment with flavopiridol and CDK9 knockdown result in reduced H1 phosphorylation (Fig. 2A,B).

Importantly, neither flavopiridol treatment nor CDK9 depletion resulted in apoptosis in C2C12 as measured by ELISA for cytochrome C release (Supporting Information Fig. 1).

Blocking H1 phosphorylation in C2C12 cells prevents muscle marker transcription

Previously, we identified the P-TEFb-specific phosphorylation site on histone H1.1. In vitro, the phosphorylation of the S183A mutant by CDK9 was diminished, while the T152A mutant was phosphorylated by CDK9 but not CDK2 (O'Brien et al., 2010). In addition, expression of S183A GFP-H1.1 in HeLa cells inhibited expression of c-Fos, Hsp70, HIV-1 transcription. We therefore wanted to test whether preventing H1 phosphorylation with these

mutants would have a negative effect on differentiation of C2C12 cells. WT, T152A, S183A, T152A/S183A, or S183E GFP-H1.1 constructs were expressed in C2C12 cells for 24 h and then cells were placed in differentiation media for 24 h. Myogenin, MCK, and MyHC mRNAs all showed a significant decrease compared to WT H1.1 when the S183A and T152A S183A mutants were expressed (Fig. 3A–C). A phosphorylation mimic mutant, S183E GFP-H1.1, showed normal induction of these muscle marker genes. Cyclophilin A expression, unlike muscle marker expression, was not affected by any of the H1.1 mutants (Fig. 3D). The GFP-H1.1 variants also expressed at similar levels (Fig. 3E).

H1 is released in a P-TEFb-specific manner from muscle marker genes during differentiation

Our previous work in HeLa and HeLa-MAGI cells suggested that H1 is released from genes during P-TEFb-regulated transcription, and that H1 phosphorylation makes it more likely to leave DNA. We wanted to test this hypothesis in the context of differentiation by performing H1 ChIP at muscle-specific marker genes in C2C12 cells. As before, we treated cells with flavopiridol overnight, then placed cells in differentiation media with flavopiridol, and H1 ChIP was performed. We quantified H1 binding to the coding regions of the myogenin, MCK, and MyHC genes by qPCR with specific primers. We found that when differentiation is induced with horse serum, there is a loss of H1 from all these marker genes (Fig. 4A). This effect is specific to induced genes, because H1 binding at the EF1-α gene remains unaffected. When cells are induced with horse serum and treated with flavopiridol, H1 binding is restored. As a control, we examined H3 binding with the same cellular conditions. Histone H3 binding, unlike H1, remains the same with horse serum or flavopiridol treatment (Fig. 4B).

Flavopiridol and CDK9 depletion inhibits C2C12 myotube formation

Next, we wanted to examine the effect of P-TEFb activity on the morphology change that C2C12 cells undergo from myoblasts to myotubes. By staining for a late marker such as myosin, we could pinpoint cells that had undergone myotube formation. Therefore, we performed immunofluorescence using an antibody directed against MyHC. By the time MyHC protein is expressed, cells are elongated into myotube form.

We also wanted to observe the effect of CDK9 inhibition on myotube formation. We treated C2C12 cells with flavopiridol or transfected them with CDK9 siRNA and induced differentiation. After changing media to horse serum, we stained for MyHC. When P-TEFB was inhibited by flavopiridol or CDK9 depletion, much fewer cells stained positive for MyHC, and the cells that did stain positive did not organize into elongated myotubes (Fig. 5A, Supporting Information Fig. 2A).

H1 phosphorylation promotes myotube formation

Because the expression of S183A GFP-H1.1 prevented the induction of muscle marker gene expression, we also wanted to determine how preventing H1 phosphorylation affects C2C12 morphology. MyHC immunofluorescence experiments were performed with C2C12 cells that were transfected with WT, T152A, S183A, T152A/S183A, or S183E GFP-H1.1. Expression of WT and T152A H1.1 results in similar MyHC expression and myotube formation, as does expression of the phosphorylation mimic, S183E H1.1. The expression of S183A and T152A/S183A H1.1, however, results a reduction in the number of both MyHC-positive cells and organized myotubes formed (Fig. 5B, Supporting Information Fig. 2B).

Discussion

The timing of gene transcription during development and differentiation of tissues needs to be tightly controlled. To prevent the aberrant growth of different tissue types, gene expression must be activated specifically. Because of its role in elongation of transcripts with poised polymerases, we wanted to explore the role P-TEFb plays in the context of muscle development and regeneration.

We found that P-TEFb inhibition, both by flavopiridol and CDK9 siRNA, decreased both early and late transcription using three muscle marker genes in C2C12 cells. After observing the dependence of muscle gene transcription on active P-TEFb, we wanted to examine the effects of P-TEFb inhibition on cell differentiation and morphology change. Flavopiridol treatment and CDK9 depletion not only inhibited the expression of MyHC in immunofluorescence experiments, but also prevented the formation of elongated, organized myotubes.

In our previous work, we characterized the phosphorylation of histone H1 by P-TEFb (O'Brien et al., 2010). We therefore examined H1 phosphorylation in C2C12 cells. In differentiating C2C12 cells, H1 phosphorylation is induced, but P-TEFb inhibition prevents this phosphorylation. These data suggest that, along with activating muscle gene transcription, P-TEFb also directs H1 phosphorylation during differentiation.

Previously, we characterized a specific site on histone H1.1 that is phosphorylated by P-TEFb. To further examine the role of H1 phosphorylation in muscle gene transcription, we expressed GFP-WT, T152A, S183A, T152A S183A, and S183E H1.1 in C2C12 cells. S183A and T152A S183A GFP-H1.1 expression prevented the induction of myogenin, MCK, and MyHC when cells were placed in differentiation media. Thus, preventing H1 phosphorylation by P-TEFb at S183 specifically inhibited transcription. H1 ChIP experiments also showed that H1 is released from these genes during differentiation, while flavopiridol treatment inhibits this release. Like our previous work, which studied H1 phosphorylation at the *c-fos* and *hsp70* genes and the HIV-1 LTR (O'Brien et al., 2010), we find that H1 needs to be phosphorylated at P-TEFb-specific sites and to dissociate from DNA in order for muscle-specific transcription to proceed. H1 phosphorylation is therefore critical to the skeletal muscle differentiation program.

Our data provide an additional role for P-TEFb in differentiation. Other groups had previously characterized P-TEFb as a necessary factor for muscle differentiation (Simone et al., 2002; Giacinti et al., 2006; Giacinti et al., 2008). Original studies in C2C12 showed that over-expression of dominant negative CDK9 prevents myotube formation (Simone et al., 2002). Similarly, we report that inhibition of CDK9 by flavopiridol and depletion by RNAi result in the same phenotype as depletion of myogenin. We additionally show that P-TEFb inhibition prevents the expression of genes specific to myotube formation and contractile function, and that H1 phosphorylation is required for the transcription of these genes.

The major muscle regulatory factor MyoD interacts with and recruits P-TEFb to the promoters of muscle-specific genes (Simone et al., 2002; Giacinti et al., 2006). P-TEFb also interacts with the MEF2 family of transcription factors, providing additional activation of muscle structural genes (Nojima et al., 2008). Once these factors recruit P-TEFb to promoters, CDK9 is then able to phosphorylate RNA Pol II and the NELFs and promote transcription elongation. Our data suggest that H1 also needs to be phosphorylated to promote the transcription of these genes. MyoD also binds the histone acetyltransferases p300 and pCAF, and their activity in turn recruits the SWI/SNF complex to remodel chromatin (Puri et al., 1997; Sartorelli et al., 1999; Giacinti et al., 2008). Although the timing of events is unclear, it is possible that phosphorylation of H1 by P-TEFb can result in

increased accessibility for these acetyltransferases and chromatin modifiers to nucleosomes. This activity would facilitate Pol II transcription through chromatin.

P-TEFb has also been implicated in muscle regeneration. In experiments using skeletal muscle tissue from mice, CDK9 mRNA and protein expression is induced in satellite cells when muscle is damaged (Giacinti et al., 2008). While we have not examined muscle regeneration in this study, we would hypothesize that H1 phosphorylation is induced during regeneration after damage as well as initial tissue differentiation.

As a chromatin structural protein, histone H1 has proven essential for proper development in mammals. As pluripotent cells differentiate, the total amount of H1 in cells increases (Fan et al., 2003; Fan et al., 2005). Likewise, higher order packing of chromatin occurs, determining the accessibility of genes for transcription. Temporary changes in H1 binding would be beneficial for timing the expression of specific genes. P-TEFB is emerging as an important factor in differentiation-specific transcription not only in myotube precursors but also in pluripotent and embryonic stem cells (Kohoutek et al., 2009; Kaichi et al., in press). Our work suggests that H1 is one substrate by which P-TEFb acts to trigger differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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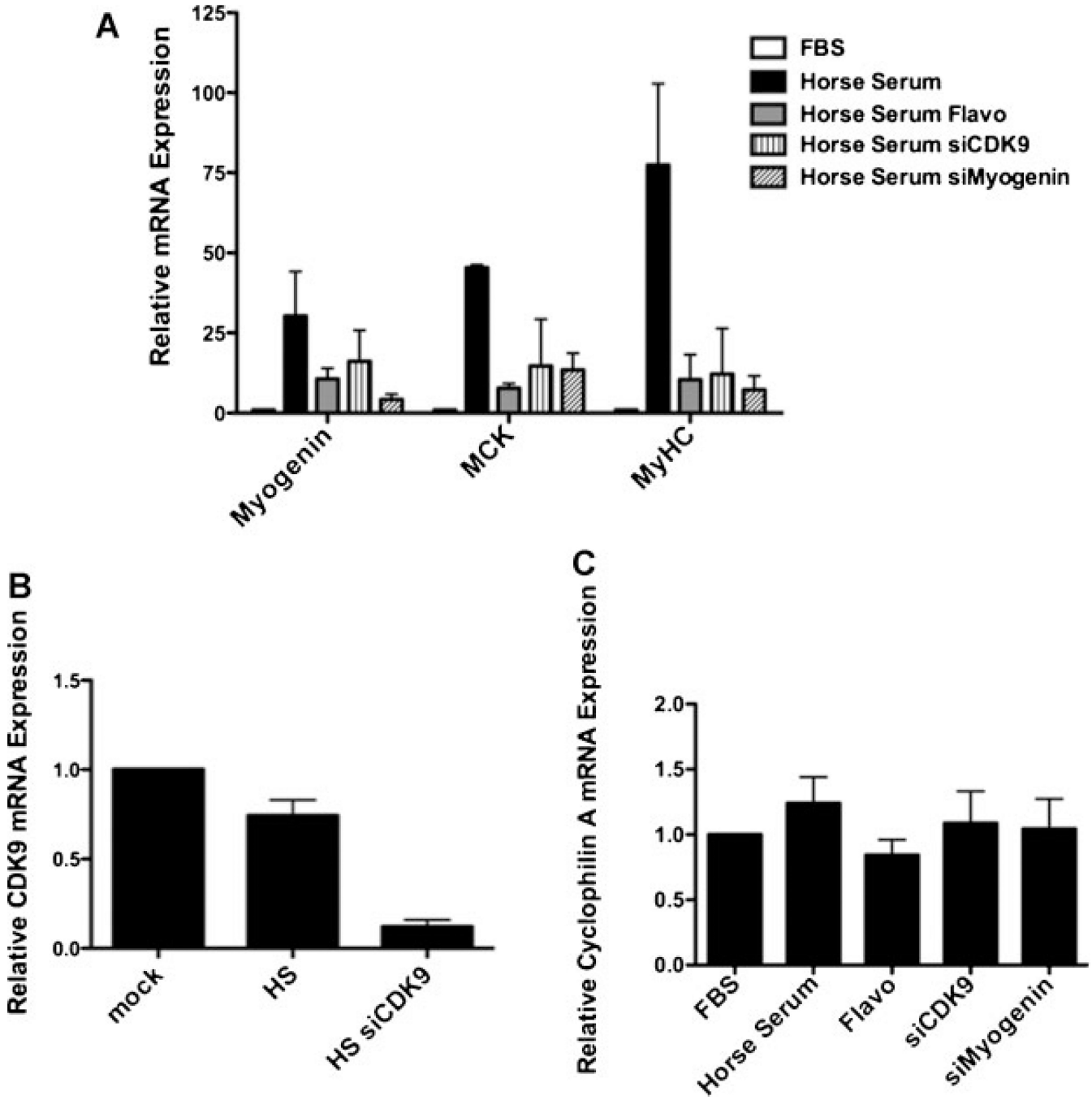

Fig. 1.

Inhibition of P-TEFb in C2C12 cells inhibits early and late muscle marker expression. A: qPCR for myogenin, MCK, and MyHC expression. Cells were incubated overnight in DMEM with 10% FBS with DMSO or 25 nM flavopiridol. For siCDK9 and siMyogenin samples, 50 nM siRNA was transfected using Lipofectamine 2000. Cells were incubated with transfection mixes overnight, and the media was replaced with differentiation media (DMEM + 2% horse serum) and flavopiridol was added again (25 nM). RNA samples were isolated 24 h later for qPCR. B: qPCR for CDK9 expression. C2C12 cells were mock transfected or transfected overnight with 50 nM CDK9 or myogenin siRNA. Cells were then

placed in differentiation media for 24 h. C: qPCR for cyclophilin A expression. C2C12 cells were treated or transfected as described above, then placed in differentiation media for 24 h.

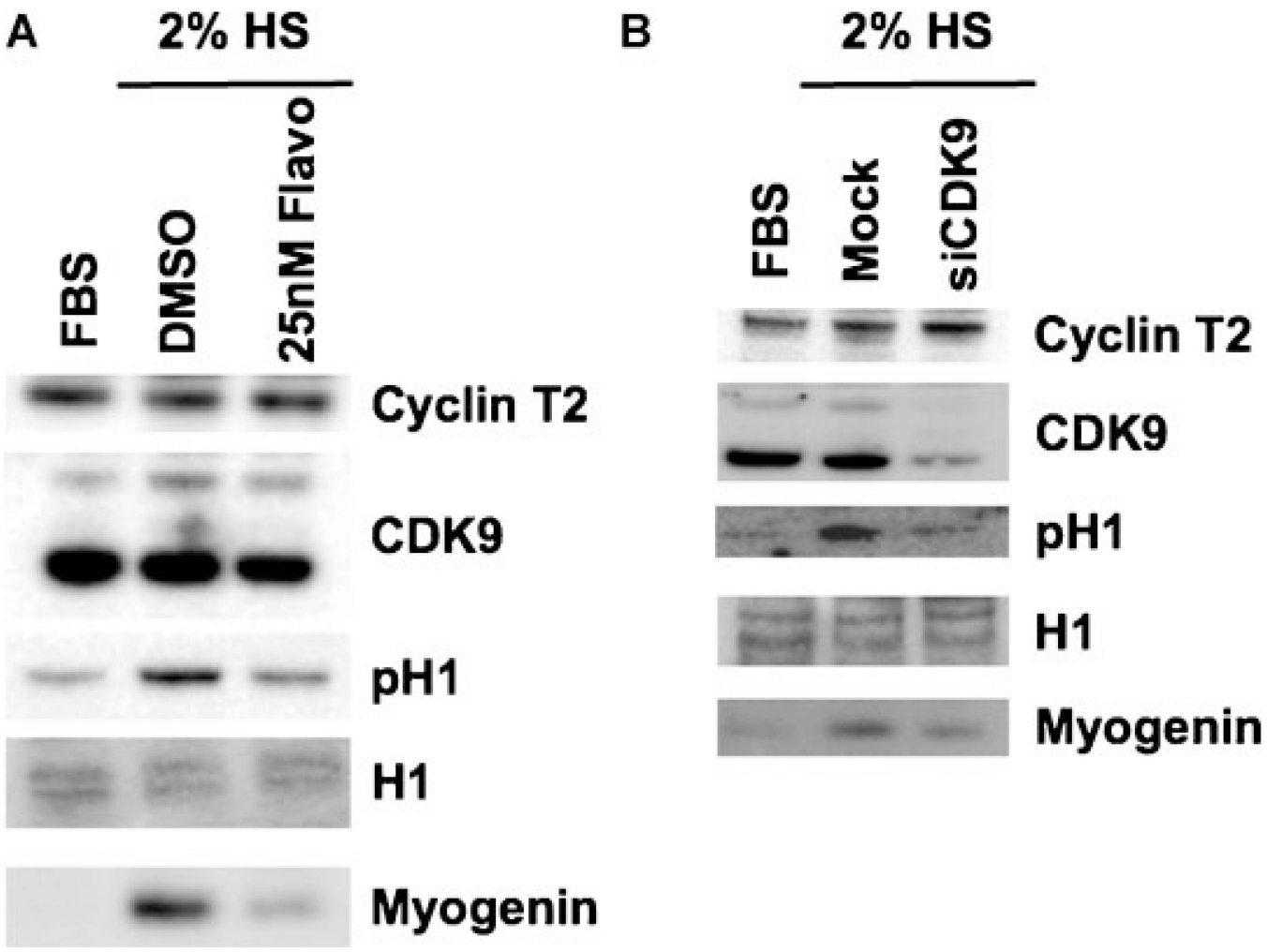

Fig. 2.

P-TEFb inhibition in differentiating C2C12 cells results in reduced histone H1 phosphorylation. A: Western blot for H1 phosphorylation in the presence or absence of Flavopiridol. Cells were differentiated and treated with DMSO or Flavopiridol, and total cell extracts were harvested. Total protein samples (5 μg) were run on 4–12% SDS–PAGE. B: Western blot for H1 phosphorylation in cells transfected with siRNA directed against CDK9. Cells were transfected with 50 nM CDK9 siRNA and then placed in differentiation media for 24 h.

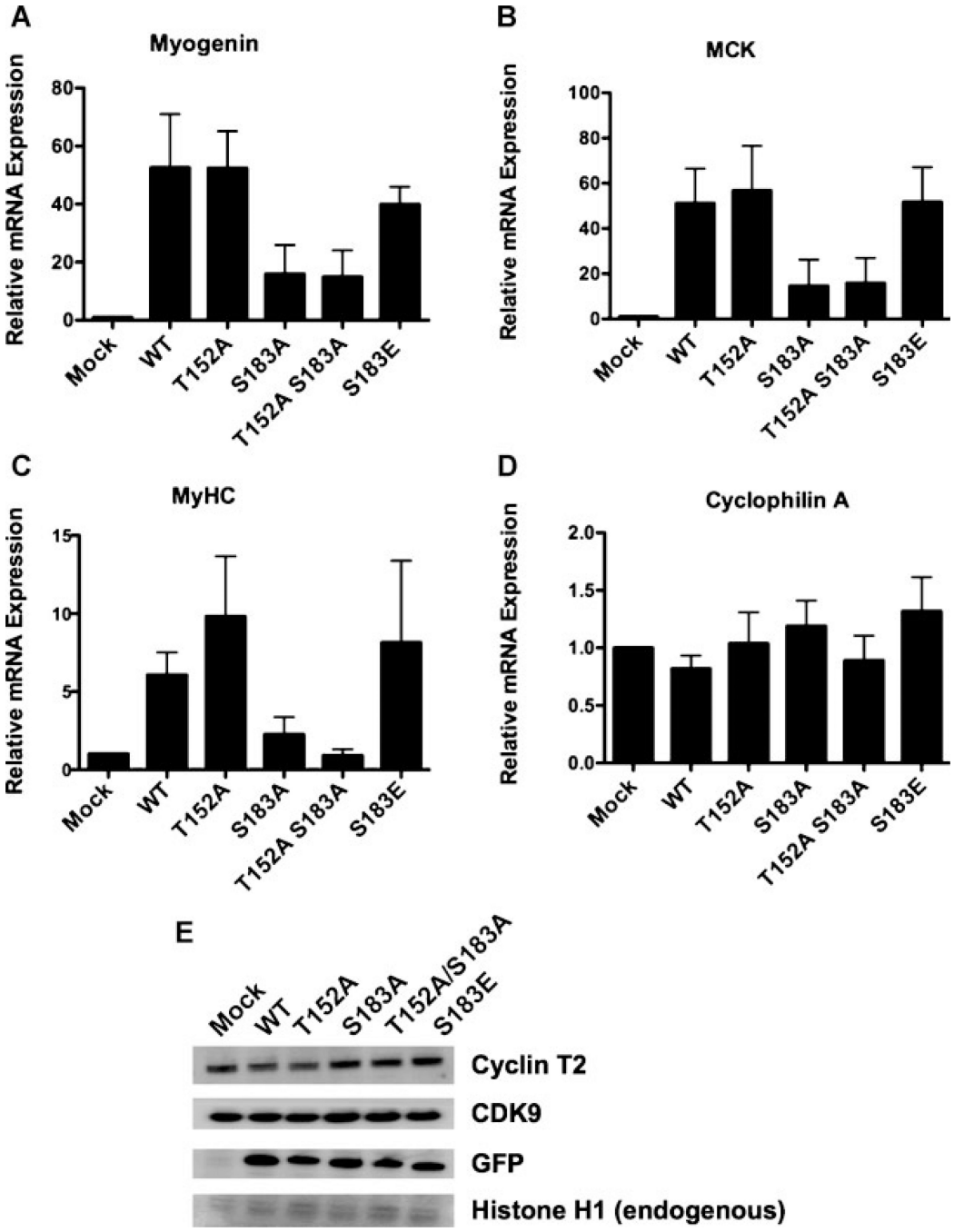

Fig. 3.

Mutation of S183 of histone H1.1 inhibits early and late muscle marker expression. C2C12 cells were transfected with WT, T152A, S183A, T152A/S183A, or S183E GFP-H1.1 constructs and were allowed to express overnight. The following day, media was changed to DMEM + 2% horse serum, and RNAs were collected 24 h later. A: Transcription of myogenin is inhibited by S183A and T152A/S183A GFP H1.1 expression. B: Transcription of MCK is inhibited by S183A and T152A/S183A GFP H1.1 expression. C: Transcription of MyHC is inhibited by S183A and T152A/S183A GFP H1.1 expression. D: Transcription of cyclophilin A is not affected by GFP-H1.1 expression in C2C12 cells. E: Western blot for GFP-H1.1 expression in C2C12 cells.

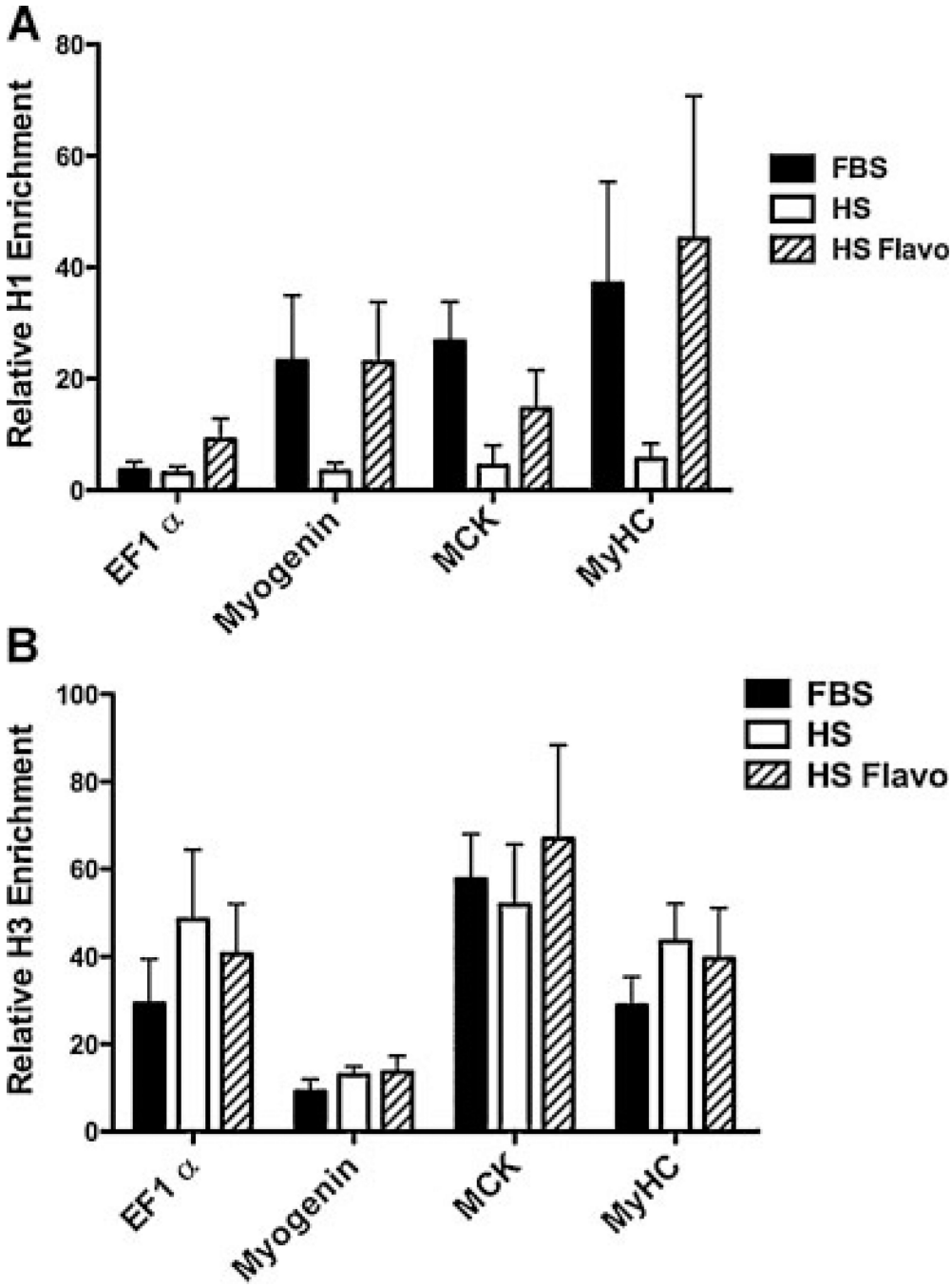

Fig. 4.

Histone H1 is released from the coding regions of muscle marker genes during differentiation in a P-TEFb-specific manner. A: H1 ChIP was performed with lysates from C2C12 cells growing in DMEM + 10% FBS, and DMEM + 2% horse serum with DMSO or 25 nM flavopiridol. qPCR of the ChIP DNA samples was performed using primers for EF1-α, myogenin, MCK, and MyHC. B: Histone H3 total binding is not affected by inhibition of P-TEFb. H3 ChIP was performed using lysates from C2C12 grown in FBS, horse serum, or horse serum with flavopiridol.

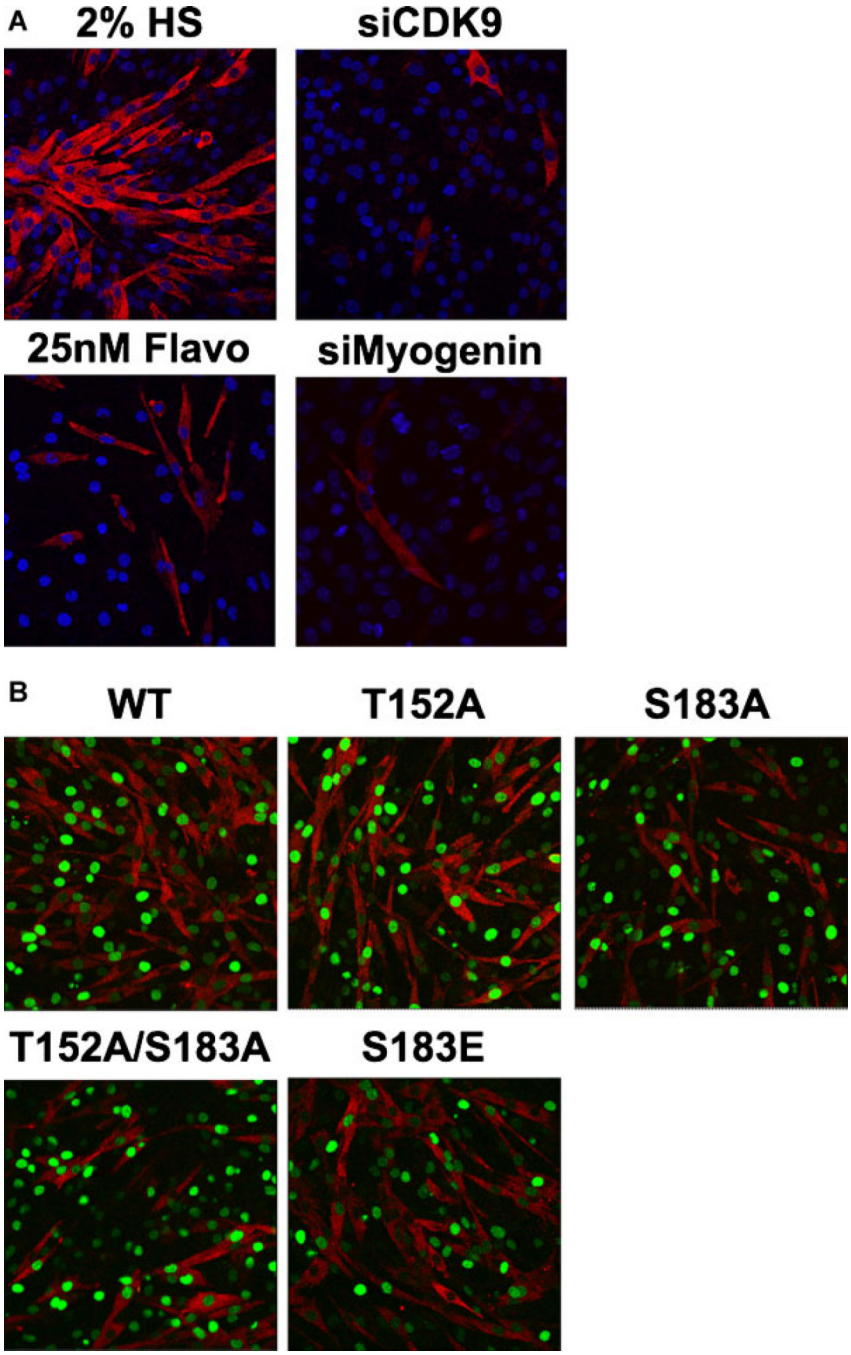

Fig. 5.

Differentiation of C2C12 cells, measured by immunofluorescence of MyHC, is inhibited by P-TEFb inhibition and S183A-H1.1 expression. A:MyHC immunofluorescence of C2C12 cells in the presence of flavopiridol or siRNA directed against CDK9 or myogenin. C2C12 cells were plated in DMEM + 10% FBS with or without 25 nM flavopiridol. The following day, media was changed to DMEM + 2% horse serum, and flavopiridol (25 nM) was added again. 48 h later, immunofluorescence was performed using an antibody directed against MyHC. Images were taken using Leica confocal microscope at 40× magnification. B: MyHC Immunofluorescence in the presence WT and mutant GFP-H1.1. C2C12 cells were transfected with WT, T152A, S183A, or T152A/S183A GFP-H1.1, and incubated overnight.

After 24 h, media was changed to 2% horse serum and the constructs were transfected again. Immunofluorescence for MyHC expression was performed 48 h later.